Journal of Microscopy, Vol. 231, Pt 1 2008, pp. 186–189
Received 26 November 2007; accepted 22 January 2008

A simple technique to minimize heat damage to specimens during thermal polymerization of LR White in plastic and gelatin capsules

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Key words. Heat polymerization, immunocytochemistry, London Resin White, preservation of anti-genicity.

Summary

London Resin (LR) White is a commonly used resin for embedding specimens to be used for immuno- and/or cytochemical studies. In some instances, due to either the properties of the specimen or the availability of various reagents and equipment, it becomes necessary and/or more convenient to polymerize LR White using heat rather than chemical accelerators or UV light. It is known, however, that heat can reduce or even eliminate the anti genicity of the tissue being embedded. It is therefore desirable to polymerize specimens at the lowest temperature possible and to remove the specimens from the oven as soon as polymerization is complete. We have developed a technique that provides a visual marker that allows the exothermic polymerization of LR White to be monitored, thus minimizing the amount of time a specimen must stay in the oven while excluding oxygen from capsules of polymerizing LR White.

Introduction

London Resin (LR) White, an acrylic resin, polymerizes via a free radical mechanism. Oxygen acts as a radical scavenger and must, therefore, be excluded during polymerization. Several methods exist for excluding oxygen from LR White during polymerization. These range from simply closing the lid of plastic or gelatin capsules, to the use of oxygen-impermeable Aclar® films, to more elaborate nitrogen-filled polymerization chambers (e.g. Vesk *et al.*, 1993; Harris *et al.*, 1995). We have found that Aclar® films work very well on large, shallow containers where the Aclar® can flex downward as the LR White shrinks during polymerization. In fact, Aclar® sheets coupled with the new 26-mm-diameter polypropylene embedding capsules (Ted Pella #21460) go a long way towards obviating the need for nitrogen-flushed polymerization chambers in order to achieve flat embedding of specimens in LR white (unpublished results). However, Aclar® does not work well on plastic or gelatin capsules, where the diameter of the container is too small for the relatively stiff sheet of Aclar® to bend down into during polymerization. This causes an air gap to form between the Aclar® and the surface of the resin as the resin shrinks, which leaves the top of the block unpolymerized.

There seems to be a general consensus in the microscopy community that LR White must be polymerized for 24–48 h at 50–65°C (e.g. Newman & Hobot, 1993; Vesk *et al.*, 1993; Harris *et al.*, 1995; Palmieri & Kiss, 2005; Microscopy Today Netnotes, March 2007, Microscopy Today Netnotes, May 2007, and many more). More specifically, the Technical Data Sheet for LR White from Polysciences (Data Sheet 305A) recommends 60–65°C for 12–20 h and the Technical Note from Ted Pella (TN 602) recommends ≥65°C for 24–48 h (these are the two brands of resin used in this study). In our lab, however, we routinely polymerize LR White at 55°C for 2–2.5 h in closed flat-bottomed polypropylene capsules (TAAB). This short polymerization time has allowed us to detect many epitopes from the cytoskeleton (Hoffman *et al.*, 1994), peroxisome (Pettigrew & Vaughn, 1998), chloroplast (Pettigrew & Vaughn, 1998) and cell walls (Bowling & Vaughn, 2008) that could not have been detected with the longer and/or higher-temperature polymerization regimes. The completion of polymerization can be judged by the mobility/immobility of the air bubble that inevitably gets trapped under the lid of plastic, BEEM-type capsules and gelatin capsules. We noticed, however, that frequently the bottom of the capsule would feel hard, as if the polymerization was complete in the lower portion of the capsule, whereas the resin around the bubble was still liquid. Thus, we began to experiment with various techniques that would eliminate even this small amount of air trapped

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within the capsule in order to reduce the time the specimen is in the oven to the absolute minimum, with the ultimate goal of improving specimen anti-genicity.

Materials and methods

Polyethylene capsules (BEEM, pyramidal tip, Ted Pella #130; TAAB, flat-bottomed capsules, Ted Pella #133), polypropylene capsules (TAAB, flat-bottomed capsules, Ted Pella #133-P) and size 00 hard gelatin capsules, with and without specimens, were filled to the top with LR White resin, medium grade (Polysciences #17411 or Ted Pella #18181). A small square (2–3 mm) of pink dental wax (Extra Tough Pink Base Plate Wax, Hygenic Corp, Akron, OH, Ted Pella #109–2) was cut with a razor blade and floated on top of the LR White (Fig. 1(A)). The lids of the capsules were left open or off. The capsules were loaded into multi-capsule trays (BEEM, Ted Pella #132) and placed into an oven set at 55°C. Paraffin wax (Paraplast, Electron Microscopy Sciences #19218) was also tested for the ability to mark polymerization or seal block away from oxygen, but at an even lower oven temperature (50°C).

Results and discussion

Acrylic resins (such as LR White) polymerize through an exothermic free radical mechanism. The polymerization of capsules of LR White resin appears to occur from the bottom up, regardless of whether there is a specimen in the capsule or not. The polymerization of the resin slows down as it approaches the surface of the resin where the oxygen level is higher due to its increased proximity to oxygen-containing air. Some air is usually trapped into the capsule when the lid is put on and some air enters as the resin shrinks during polymerization. We tested several different compounds for the ability to seal the resin away from oxygen, and found dental wax to be ideal for this purpose. Furthermore, we found that there was no need to pre-melt the wax to seal the block completely. A relatively small (2–3 mm) square of dental wax was floated on the surface of the resin (Fig. 1(A)) and, after approximately $1^{1}/_{4}$–$1^{3}/_{4}$; h in the oven, the wax square melted and covered the block, thus sealing the block away from oxygen and moving with the retreating surface of the LR White to maintain an air-tight seal. The melted dental wax appears as a clear liquid that remains floating on top of the resin. The presence of this clear layer is brief: after only 5–10 min, the block cools sufficiently to allow the wax to re-solidify. The re-solidified wax layer now appears white (Fig. 1(B)) and is a sign that the polymerization is complete.

We found that dental wax was ideal for this purpose for several reasons, the primary reason being that this wax does not melt at the temperature of the oven but only melts when the temperature of the resin rises during polymerization, so that the wax also serves as a marker for the completion of polymerization. This allows specimen blocks to be removed from the oven as soon as polymerization is

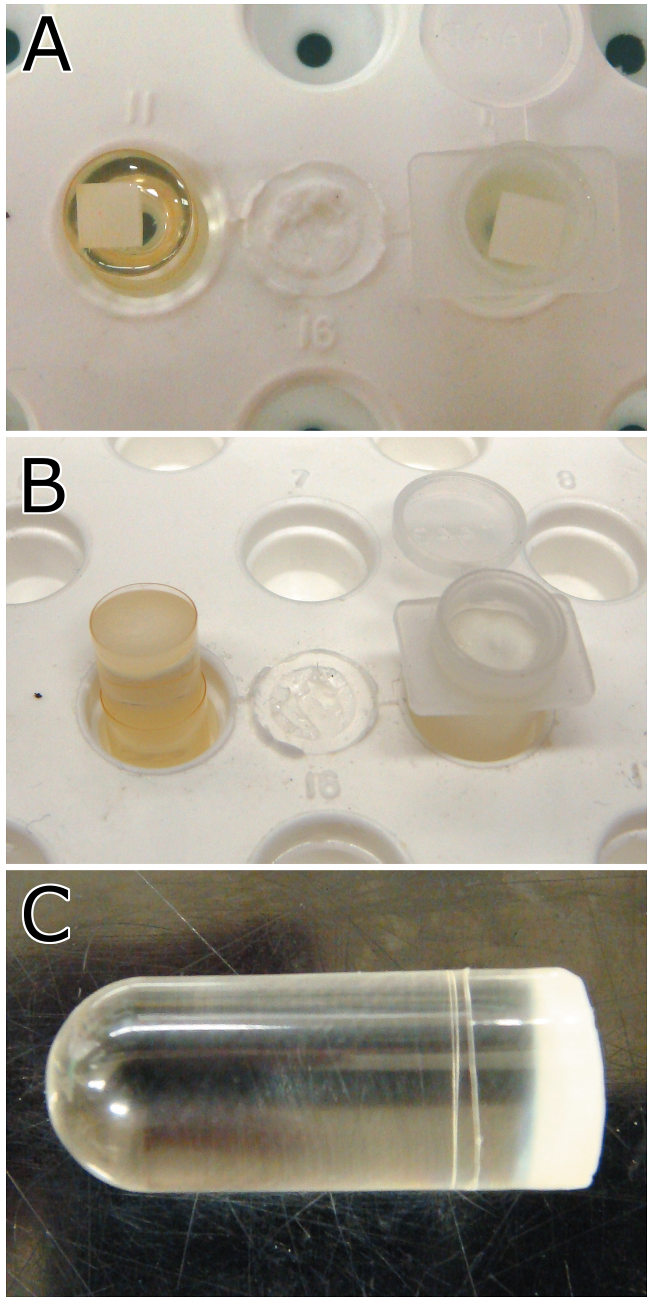

Fig. 1. The set-up and results of the wax-sealed capsule technique. (A) A gelatin capsule (left) and a polyethylene capsule (right) both filled with LR White resin, with a square of dental wax floating in them. (B) After 1.75 h at 55°C, the resin has polymerized and the wax squares have melted, forming a complete white layer at the top of the capsules. (C) A representative block after removal from the gelatin capsule. A razor blade was pressed against the capsule and used to roll the capsule across the bench, cutting a circumferential line just below the wax layer. The bottom of the gelatin capsule was slid off of the end of the block and the remaining ring was removed upwards.

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complete, minimizing the exposure of the specimen to heat. This technique is very simple, low-cost and requires no special equipment (vacuum ovens or custom-made gas-purgeable chambers), or cylinders of dry purge gas.

The effect of capsule material on the polymerization of LR White

Polymerization, marked by the formation of the white, re-solidified wax layer, appears to occur sooner in gelatin and polypropylene capsules than in polyethylene capsules. In fact, the Technical notes from Ted Pella state that polymerization in gelatin capsules can be done at 50°C, whereas polymerizations in polyethylene BEEM-type capsules must be done at temperatures ≥65°C. This difference in polymerization rates at a given temperature may be due to the greater permeability of polyethylene to oxygen (Newman & Hobot, 1993), which would act to quench the initial formation of free radicals in the resin and thus delay the onset of the exothermic chain reaction. It is likely that this is the reason that blocks polymerized in polyethylene capsules are sometimes slightly softer than blocks polymerized in polypropylene and gelatin capsules.

Polymerization at 50°C using paraffin

To minimize heat damage to the specimen, it is important to use the lowest possible temperature for polymerization. Furthermore, polymerization of LR White at 50°C for 24 h was advocated by Newman & Hobot (1993) for the purpose of reducing the cross linking of the resin in such a manner as to facilitate the penetration of sections by aqueous reagents. Unfortunately, below 55°C, the exothermic polymerization reaction does not reach a high enough temperature to melt the wax and cover the block. Paraffin is another wax that is commonly found in histology labs, and with a melting point of 56°C, it would seem to be ideal for polymerizations at 50°C. However, using Paraplast™ wax, we found that the blocks were largely unpolymerized after several hours or even overnight. With further study, we found that in a glass Petri dish at 50°C, paraffin melts slightly but not completely, where dental wax does not melt at all, even at 55°C. It appears that at 50°C, the paraffin melts slightly and mixes with the resin and interferes with subsequent polymerization. In fact, the reason that dental wax works so well for this purpose may be its more well-defined melting point. As previously mentioned, polymerization always seems to begin in the bottom of the capsule, where most fully infiltrated specimens will be located. By the time the temperature rises high enough to melt the dental wax, the resin containing the specimen has already been polymerized. The fact that the dental wax used in this technique has a well-defined melting point means that the wax molecules are physically precluded from mixing with the resin until the lower portion of the resin (containing the specimen) has already been polymerized and only the upper portion remains. There may be other waxes besides dental wax that will allow polymerization at 50°C, but they would need to have an appropriate melting point (between 55°C and 60°C) and they should melt uniformly over a small temperature range so as to minimize the chances of mixing with the resin or, alternatively, they must be totally insoluble in LR White resin.

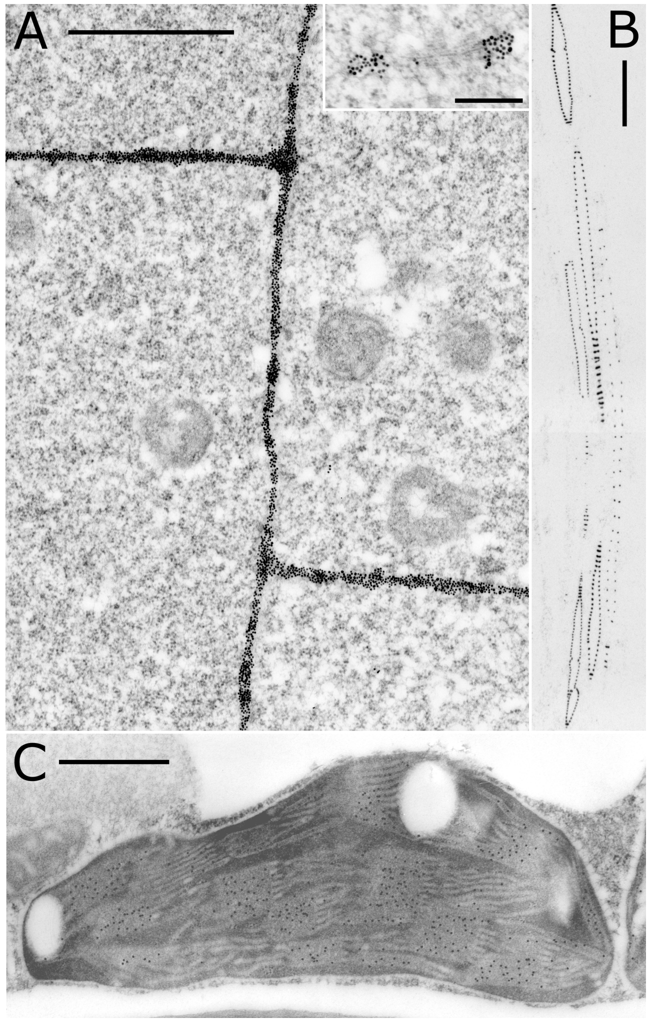

Fig. 2. A sample of specimens labelled immunocytochemically following a 2-h heat polymerization. (A) An *Arabidopsis* root labelled with an antibody raised to seed coat mucilage (CCRC-M38). The cell wall can be seen to be very heavily labelled with this antibody. Scale bar = 1 μm. Inset: At higher magnification, the synthesis of this polysaccharide in the Golgi is clearly visible. Scale bar = 0.5 μm. (B) A stem from *Impatiens* labelled with an anti-xylan antibody (LM10) and silver-enhanced for observation by light microscopy. The thickenings of the xylem are decorated very specifically. Scale bar = 100 μm. (C) A chloroplast from cotton leaf labelled with an anti-photosystem II light-harvesting complex antibody. The localization of the PS II light-harvesting complex in the stacked membranes of the grana is evident. Scale bar = 1 μm.

Possible advantages over conventional methods

Other researchers have reported that specimens processed at 55°C (as opposed to 60°C or even 65°C) for 48 h have

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increased anti genicity over specimens polymerized at higher temperatures (Harris *et al.*, 1995). Here, using the melting of the wax as a marker for polymerization, it has been possible to determine that polymerization is essentially complete after only 1.5–2.0 h in a 55°C oven. This short exposure of the specimen to a relatively low level of heat should have the benefit of further preserving the anti genicity of the tissue. The excellent preservation of antigens following this short heat polymerization is demonstrated in Fig. 2. We have used this technique extensively for localizing a wide variety of cell wall polysaccharides, including mucilage (Fig. 2(A)) and xylan (Fig. 2(B)) and many others (Bowling & Vaughn, 2008). We have also successfully immunolocalized proteins such as the light harvesting complex of photosystem II (Fig. 2(C); Pettigrew & Vaughn, 1998). In addition, when the blocks are cut from the capsules (Fig. 1(C)), the layer of wax can be left in place or trimmed off of the block with a razor blade. This has the further advantage that the end of the block is flat (as opposed to the uneven surface that results from the bubble trapped under the lid), so that the block can stand upright under the dissecting microscope for specimen selection, etc.

As mentioned previously, it takes 2.5–3.0 h for blocks to cure with closed lids, as determined by whether the bubble inside the lid moves when the capsule tray is tilted. It is unclear whether the wax truly allows for faster polymerization of the resin than in the normal closed-lid system or if the bottom of the capsules polymerize at the same rate in both systems, but that in the closed-lid system, the polymerization slows down as it approaches the top of the capsule where more O_2 is encountered from both the trapped bubble and the lid seal. In conclusion, by floating a small square of dental wax on the surface of capsules of LR White, the exothermic polymerization can be followed visually, allowing specimens to be removed from heat as soon as polymerization is complete and thus minimize the exposure of the samples to heat.

Acknowledgements

Andrew Bowling was supported by a headquarters-funded Research Associate Program grant to Kevin Vaughn. Mention of a trademark, vendor or proprietary product does not constitute an endorsement by the USDA.

References

Bowling, A.J. & Vaughn, K.C. (2008) Structural and immunocytochemical characterization of the adhesive tendril of Virginia Creeper [*Parthenocissus quinquefolia* (L.) Planch.]. *Protoplasma* **232**, 153–163.

Harris, K.F., Pesic-Van Esbroeck, Z. & Duffus, J.E. (1995) Moderate-temperature polymerization of LR White in a nitrogen atmosphere. *Microsc. Res. Tech.* **32**, 264–265.

Hoffman, J.C., Vaughn, K.C. & Joshi, H.C. (1994) Structural and immunocytochemical characterization of microtubule organizing centers in pteridophyte spermatogenous cells. *Protoplasma* **179**, 46–60.

Newman, G.R. & Hobot, J.A. (1993) *Resin Microscopy and on-Section Immunocytochemistry*. Springer-Verlag, Berlin.

Vesk, P.A., Dibbayawan, T.P. & Vesk, M. (1993) Rapid communication: flat embedding of microorganisms on slides with LR white for immunocytochemistry. *Microsc. Res. Tech.* **26**, 352–353.

Palmieri, M. & Kiss, J.Z. (2005) A novel technique for flat-embedding cryofixed plant specimens in LR white resin. *Microsc. Res. Tech.* **68**, 80–84.

Pettigrew, W.T. & Vaughn, K.C. (1998) Physiological, structural, and immunological characterization of leaf and chloroplast development in cotton. *Protoplasma* **202**, 23–37.

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